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A Dominant-Negative GFI1B Mutation in the Gray Platelet Syndrome

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SUMMARY

The gray platelet syndrome is a hereditary, usually autosomal recessive bleeding disorder caused by a deficiency of alpha granules in platelets. We detected a nonsense mutation in the gene encoding the transcription factor GFI1B (growth factor independent 1B) that causes autosomal dominant gray platelet syndrome. Both gray platelets and megakaryocytes had abnormal marker expression. In addition, the megakaryocytes had dysplastic features, and they were abnormally distributed in the bone marrow. The GFI1B mutant protein inhibited nonmutant GFI1B transcriptional activity in a dominant-negative manner. Our studies show that GFI1B, in addition to being causally related to the gray platelet syndrome, is key to megakaryocyte and platelet development.

Platelets are formed through fragmentation of megakaryocytes that reside in the bone marrow. Platelet alpha granules, which are by far the most abundant platelet organelles, store proteins that stimulate platelet adhesiveness, hemostasis, and wound healing. The gray platelet syndrome is an inherited bleeding disorder characterized by defective production of alpha granules. Patients with this syndrome have reduced numbers of larger-than-normal platelets, and on light microscopy these platelets have a typical gray appearance caused by the lack of alpha granules. For a final diagnosis, the lack of alpha granules must be confirmed by means of electron microscopy. Clinically, patients with the gray platelet syndrome have bleeding tendencies with variable severity. Additional features associated with this syndrome are bone marrow fibrosis and the capture of neutrophils within megakaryocytes in a process known as emperipolesis (the presence of an intact cell within the cytoplasm of another intact cell).

It has been proposed that the alpha-granule deficiency in the gray platelet syndrome can be attributed to the failure of megakaryocytes to efficiently route endogenous proteins into alpha granules, thereby hampering the maturation of these granules. Although the gray platelet syndrome is most often an autosomal recessive disease, families with an autosomal dominant inheritance pattern have been described as well. Recently, biallelic NBEAL2 missense mutations have been

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identified in an autosomal recessive form of the gray platelet syndrome.\textsuperscript{12-14} NBEAL2 localizes to the dense tubular system in platelets,\textsuperscript{12} but how NBEAL2 mutations contribute to defective alpha-granule formation remains unknown. No genes have been implicated in autosomal dominant gray platelet syndrome. Here we report on the identification of a nonsense mutation in the transcription-factor gene, growth factor independent 1B (GFI1B), in a family with autosomal dominant gray platelet syndrome. GFI1B and its paralog GFI1 function as transcriptional repressors and play fundamental roles during hematopoiesis.\textsuperscript{15-20} Both proteins contain a highly conserved C-terminal region that includes three zinc fingers that are necessary for interaction with DNA and an identical N-terminal region through which epigenetic modifiers required to inhibit transcription are recruited.\textsuperscript{16,21-23} In addition, GFI1 and GFI1B interact directly with other transcription factors to coregulate gene expression during blood-cell development.\textsuperscript{15}

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METHODOLOGY

STUDY FAMILY

We studied a large family with an autosomal dominant form of thrombopathic thrombocytopenia characterized by mild-to-severe bleeding complications, a diagnosis originally described in 1968.\textsuperscript{24} The thrombopathic disorder in this family consisted of a low platelet count, an increased platelet size, and few alpha granules. An updated evaluation of affected family members according to the current disease classification allowed us to redefine the clinical diagnosis as the gray platelet syndrome (number 139090 in the Online Mendelian Inheritance in Man database).

The study was approved by the institutional review board of Radboud University Medical Center. Written informed consent was obtained from all family members who underwent evaluation.

LABORATORY ANALYSES

The bleeding score was calculated, and morphologic and immunohistochemical studies, electron microscopy, flow cytometry, GFI1B sequencing, and platelet-related analyses were performed according to standard procedures (see the Supplementary Appendix, available with the full text of this article at NEJM.org). Measurements of plasma glycoprecipitin (Cusabio) and megakaryocyte colony-forming assays (StemCell Technologies) were performed according to the manufacturers’ instructions. Linkage analysis based on genome-wide single-nucleotide polymorphism array with the use of Linkage 24 DNA Analysis BeadChips (Illumina), GFI1 and GFI1B gene-reporter assays, the method of retroviral transduction, and primary cell cultures are described in the Supplementary Appendix.

RESULTS

CLINICAL FINDINGS AND LABORATORY ANALYSES

Morphologic analysis of blood smears from the six affected family members who were evaluated showed a ghostlike, gray appearance of enlarged platelets, in contrast to the normal platelets observed in the two unaffected family members (Fig. 1A and 1B). Electron-microscopic analysis confirmed a marked reduction in the number of alpha granules (Fig. 1C, 1D, and 1E).

Affected family members had moderate-to-
severe bleeding tendencies (Table S1 in the Supplementary Appendix). In addition to reduced platelet counts, diminished platelet factor 4 and β-thromboglobulin levels were observed in platelets obtained from the affected family members (Table S1 in the Supplementary Appendix).

**Histopathological Findings**

Histopathological examination of a bone marrow biopsy specimen obtained from Family Member III.2 (Fig. 2A) showed stage I myelofibrosis (Fig. 1F) and cellular marrow with an increased number of megakaryocytes that were pleomorphic in size and shape (Fig. 1G, and Fig. S1 in the Supplementary Appendix). Emperipolesis was frequently observed (Fig. 1H, and Fig. S1 in the Supplementary Appendix). Megakaryocytes were clustered along bone marrow sinuses and had stretched features (Fig. 1I, and Fig. S1 in the Supplementary Appendix).

**Linkage and Sequence Analyses**

To determine the disease-causing mutation, we first performed linkage analyses in 14 family members and identified a candidate locus on chromosome 9q34 with a maximum LOD score of 3.9 (Fig. S2 and S3 in the Supplementary Appendix). Within the linked region, we considered GFI1B to be an excellent candidate gene because it encodes a transcriptional repressor that has been implicated in megakaryopoiesis.17-19

Sequence analysis detected a nonsense mutation in exon 6 (c.859C→T, p.Gln287*) of GFI1B that completely cosegregates with the gray platelet syndrome (Fig. 2A and 2B, and Fig. S4 and Table S2 in the Supplementary Appendix). The mutation introduces a premature stop codon that is predicted to lead to the formation of a truncated protein (GFI1BTr) that lacks 44 carboxy-terminal amino acids (Fig. 2B). Since premature stop codons can induce nonsense-mediated messenger RNA (mRNA) decay, we determined whether mutated GFI1B was expressed at the mRNA level. Sequencing of complementary DNA generated from CD34+ progenitor cells obtained from two affected family members showed that mutated and nonmutated GFI1B transcripts were expressed, indicating that the mutated transcript is not targeted for decay (Fig. S4 in the Supplementary Appendix).

**Molecular and Functional Characterization of Mutated GFI1B**

GFI1B functions as a transcriptional repressor. The truncating mutation is located within zinc finger 5, which is required for DNA binding. This zinc finger is 100% identical between humans and rodents (rats and mice), and it is identical to zinc finger 5 of the paralog GFI1 (Fig. 2B).20 Murine Gfi1 and Gfi1b bind the same DNA consensus sequence, and Gfi1 zinc finger 5 directly interacts with the major groove of the DNA core sequence AATC through four amino acids.22 Because the truncated protein GFI1BTr lacks all these DNA-interacting amino acids (Fig. 2B), we hypothesized that it would be unable to repress gene expression. To test this hypothesis, we introduced the truncating mutation in a GFI1B expression vector and performed transcriptional
In repression assays using the Gfi1 promoter as a validated Gfi1B target, in line with the lack of an intact DNA-binding zinc finger, we observed that GFI1BTr did not repress the Gfi1 promoter, whereas the nonmutant GFI1 and GFI1B did (Fig. 2C, and Fig. S5 in the Supplementary Appendix). When coexpressed, GFI1BTr inhibited repression mediated by nonmutant GFI1B, indicating that the mutant interferes with nonmutant GFI1B in a dominant-negative fashion (Fig. 2C).

To validate that GFI1BTr adversely affects normal GFI1B, we expressed GFI1BTr in mouse bone marrow cells. Megakaryocytes derived from GFI1BTr-expressing mouse bone marrow cells showed a reduction in luciferase activity compared to controls (Fig. D).

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A Pedigree of Family with the Gray Platelet Syndrome

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B Location of the GFI1B Mutation

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C GFI1BTr Inhibiting Nonmutant GFI1B in a Dominant-Negative Manner

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D Megakaryocytes Derived from GFI1BTr-Expressing Mouse Bone Marrow Cells

Control Vector vs GFI1BTr

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repression assays using the Gfi1 promoter as a validated Gfi1 target. In line with the lack of an intact DNA-binding zinc finger, we observed that GFI1BTr did not repress the Gfi1 promoter, whereas the nonmutant GFI1 and GFI1B did (Fig. 2C, and Fig. S5 in the Supplementary Appendix). When coexpressed, GFI1BTr inhibited repression mediated by nonmutant GFI1B, indicating that the mutant interferes with nonmutant GFI1B in a dominant-negative fashion (Fig. 2C).

To validate that GFI1BTr adversely affects normal GFI1B, we expressed GFI1BTr in mouse bone...
marrow cells and then induced megakaryocytic differentiation. GFI1B−/−-positive megakaryocytes had several dysplastic features, including hypolobulation of the nuclei, irregular contours, and multiple separated nuclei; these features were not observed in control cells (Fig. 2D, and Fig. S6 in the Supplementary Appendix). These abnormalities are very similar to those observed in cells obtained from affected family members with the gray platelet syndrome (Fig. 3E), indicating that expression of GFI1B−/− is sufficient to cause megakaryocytic abnormalities and that it functions in a dominant-negative manner.

**CELLULAR ANALYSES**

Studies involving Gfi1 knockout mice have shown that Gfi1b plays an important role in the development of several hematopoietic lineages. In humans, GFI1B is required for in vitro differentiation of erythrocytic and megakaryocytic lineages. We observed that megakaryocyte colony-forming cells were significantly more frequent in a bone marrow specimen from affected family members than in a specimen from a healthy person. In addition, the colonies from the affected family member were larger than those from a healthy control (Fig. 3A, and Fig. S7 in the Supplementary Appendix); this is in line with the increased number of megakaryocytes in the biopsy specimen from affected Family Member III.2 (Fig. 1I).

To determine whether GFI1B−/− was associated with other hematopoietic abnormalities, we performed immunophenotypic analyses of peripheral-blood specimens obtained from eight family members and five healthy controls. Myeloid and erythroid lineages were unaffected (data not shown). The surface expression of characteristic platelet markers, such as β3 glycoprotein–CD36, αIIβ integrin–CD41, and β3 integrin–CD61, was also unperturbed in all tested persons. However, as compared with unaffected family members and healthy controls, five of six affected members had a marked decrease in the level of platelet surface-membrane glycoprotein 1bα–CD42B within the αIIβ integrin–CD41 platelet compartment (Fig. S8 in the Supplementary Appendix). In addition, strong expression of CD34, which is usually confined to immature hematopoietic progenitors, was detected on platelets from all affected family members in the study (Fig. 3B).

Because platelets are derived from megakaryocytes, we evaluated whether aberrant expression of surface molecules was also present on megakaryocytes. Immunostaining (with β3 integrin–CD61) of a bone marrow–biopsy specimen obtained from affected Family Member III.2 confirmed the presence of megakaryocytes that had high levels of CD34 expression (Fig. 3C). Electron-microscopic analysis of the same biopsy specimens showed megakaryocytes with few alpha granules, which were small, irregularly shaped, and centrally located; the megakaryocytes were characterized by an extensive peripheral cytoplasm with irregular proplatelets, largely devoid of cell organelles (Fig. 3D, and Fig. S9 in the Supplementary Appendix). Consistently, bone marrow smears obtained from the same patient (Patient III.2) and her mother (Patient II.2) showed dysplastic megakaryocytes that were pale, as a result of diminished alpha granules (Fig. 3E). To determine whether these abnormalities were intrinsic to the cell, we stimulated CD34+ cells obtained from affected Family Members II.2 and III.2 to differentiate along the megakaryocytic lineage in culture. Megakaryocytic cells had dysplastic features such as those observed in the bone marrow aspirates (Fig. 3E). In addition, altered expression of CD34 and glycoprotein 1bα–CD42B was observed in megakaryocytic cells that were positive for αIIβ integrin–CD41 (Fig. S10 in the Supplementary Appendix).

**DISCUSSION**

The gray platelet syndrome is a hereditary bleeding disorder characterized by platelets that lack alpha granules. Recently, mutations in NBEAL2 have been shown to cause an autosomal recessive form of the gray platelet syndrome. We found that a mutation in GFI1B was causally related to autosomal dominant gray platelet syndrome.

In addition to large gray platelets that are almost devoid of alpha granules, other phenomena associated with the gray platelet syndrome, such as thrombocytopenia, emperipolisis, mild myelofibrosis, and low expression of platelet factor 4, were observed in affected persons. However, we also found new features of the gray platelet syndrome such as megakaryocytes and platelets that were strongly positive for the stem-cell and progenitor-cell marker CD34, and had a
Figure 3. GF11BΔ-associated Platelet and Megakaryocyte Abnormalities.

Panel A (αIIβ integrin–CD41 staining) shows the results of a megakaryocyte colony-forming assay of bone marrow cells from Family Member III.2, as compared with normal bone marrow cells from a healthy control. The cells from affected Family Member III.2 developed into more and bigger colonies. CFU denotes colony-forming units, and GPS the gray platelet syndrome. The graph in Panel B shows that platelets obtained from affected Family Members II.2, II.3, II.6, II.8, II.10, and III.2, as compared with platelets obtained from unaffected Family Members II.4 and II.7 and healthy controls, had high levels of CD34 expression. MFI denotes mean fluorescence intensity. In Panel C, CD34 staining of a bone marrow–biopsy specimen obtained from affected Family Member III.2 (left) shows a high level of CD34 expression on affected megakaryocytes. β3 integrin–CD61 staining of a subsequent section from the same biopsy specimen (right) was performed to confirm that the megakaryocytes were CD34-positive. In Panel D, an electron micrograph of a biopsy specimen obtained from Family Member III.2 shows a megakaryocyte containing a few poorly developed alpha granules (arrows). Panel E (May–Grünwald-Giemsa staining) shows a normal megakaryocyte (upper left) in a bone marrow aspirate (lower left), as compared with examples of pale dysplastic megakaryocytes generated in an ex vivo culture (lower left), which are characterized by nuclear hypolobulation and pale cytoplasm.
diminished expression of glycoprotein 1bα–CD42B. Diminished glycoprotein 1bα–CD42B platelet expression may contribute to the pathogenesis of the gray platelet syndrome, since glycoprotein 1bα–CD42B plays an important role in the initial adhesion of platelets to vascular subendothelium after injury. Low platelet expression of glycoprotein 1bα–CD42B may be related to the large platelets observed in affected persons, since large platelets are present in the Bernard–Soulier syndrome, a bleeding disorder caused by GP1BA/CD42B mutations. In addition, and possibly consequential to aberrant marker expression, we observed that megakaryocytes were abnormally distributed within the bone marrow, and they were frequently aligned in clusters along the sinuses in a stretched fashion. These features, in combination with the presence of morphologic and nuclear abnormalities in megakaryocytes, indicate that GFI1B\textsuperscript{Tr} affects the terminal maturation of megakaryocytes.

GFI1B silencing and Gfi1b ablation studies have shown that the protein is fundamental to the development of several blood-cell lineages.\textsuperscript{15,17-20} In the affected members of the family we studied, the presence of GFI1B\textsuperscript{Tr} was associated with major abnormalities only in the megakaryocytic lineage. The mutation in the gray platelet syndrome introduces a stop codon that results in a truncated GFI1B protein. The deleted region includes four amino acids that directly interact with DNA. Indeed, GFI1B\textsuperscript{Tr} expression did not inhibit gene expression, but it inhibited the function of nonmutant GFI1B. This indicates that the gray platelet syndrome may be caused not by GFI1B haploinsufficiency but rather by dominant-negative inhibition of the nonmutant protein. Indeed, forced GFI1B\textsuperscript{T} expression in normal bone marrow cells resulted in severely dysplastic megakaryocytes that were similar to those observed in affected persons. These findings are consistent with mouse models showing that only complete Gfi1b ablation disturbs in vitro development of megakaryocytes, whereas the loss of a single Gfi1b allele is not sufficient to yield a clear phenotype.\textsuperscript{17} GFI1B\textsuperscript{T} retains several corepressor and transcription factor–interacting domains and may act in a dominant-negative manner by quenching proteins that would normally cooperate with nonmutant GFI1B. The identification of GFI1B target genes that may be disturbed by GFI1B\textsuperscript{T} would provide relevant insight into the molecular pathways that are key for megakaryopoiesis and platelet production.

In summary, our data indicate that GFI1B is a causative gene in autosomal dominant gray platelet syndrome. The truncated GFI1B mutant acts in a dominant-negative manner over the nonmutant protein and affects the normal development of megakaryocytes and platelets, reflecting the pivotal role of GFI1B in governing normal megakaryopoiesis and platelet production.

Disclosure forms provided by the authors are available with the full text of this article at NEJM.org.

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